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Cellulase production by *Aspergillus Niger, Trichoderma Harzianum* and *Penicillium Chrysogenum* using some selected agricultural wastes

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ABSTRACT

With increasing cost of production in manufacturing industries, coupled with unending accumulation of organic wastes in the environment, researchers are faced with challenge of search for cheap raw materials and enzyme hyper-producing microorganisms for the production of various enzymes used in different sectors of manufacturing processes. It was with this objective that this research investigated the suitability of using some selected agricultural wastes (wastepaper, sugarcane bagasse and wheat bran) as substrates for cellulase production using some fungal species. Three fungal species including *Aspergillus niger, Trichoderma*

harzianum and *Penicillium chrysogenum* were isolated from different sources and screened for cellulase production. Cellulase producing species were inoculated into basal media containing one of the substrates as sole carbon source and incubated for 216 hours at 30°C. Cellulase activity was monitored every 24 hours and was shown to reach its peak between 96 and 144 hours. *A. niger* was observed to produce highest (5.36IU) amount of cellulase enzyme after 96 hours using wheat bran, followed by *T. harzianum* (3.62IU) and *P. chrysogenum* (3.62IU) using the same substrate. Paper was shown to support less cellulase production using all the fungal species except *A. niger* where the highest activity (1.38IU) observed was greater than that of sugarcane bagasse (0.97IU). *T. harzianum* was shown to produce less cellulase compared to other species using paper and wheat bran but performed better using sugarcane bagasse. This study therefore, revealed that the agricultural wastes used could be excellent raw materials for cellulase production and *Aspergillus niger* and *P. chrysogenum* could be better candidates using wheat bran and sugarcane bagasse.

Keywords: *Aspergillusniger, Trichodermaharzianum, Penicilliumchrysogenum,* sugarcanebagasse, wheat bran

1. INTRODUCTION

Agricultural and industrial wastes are among the causes of environmental pollution; their conversion into useful products may ameliorate the problems they cause. These wastes which include cereals, straws, leaves and corncobs etc. are highly underutilized in Africa particularly Nigeria. In most parts of the country, these materials are mainly use as animal feeds. A large quantity is left on farmlands to be decomposed by microorganisms such as bacteria and fungi [1]. Grasses, trimming of lawns and other agricultural wastes, industrial, domestic, food and urban solid waste are produced at a rate of more than 43 million tonnes per year worldwide. Utilization by recycling of these wastes would not only aid in pollution abatement but can also serve as vital sources of energy [2].Enormous amounts of agricultural, industrial and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes [3].

Cellulose, a polymer of glucose, is the most common organic compound on earth. About 33% of all plant matter is cellulose (the cellulose content of cotton is 90% and that of wood is 40 – 50%). As a polymer of glucose residues connected by β-1, 4 linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature [4]. It forms insoluble, crystalline microfibrils which are highly resistant to enzymatic hydrolysis [5]. Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources.

Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. This enzyme system includes three types of cellulases *Endoglucanases*(EG, endo-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4): hydrolyses, at random, β -1,4 glucosidic bonds at internal amorphous sites in the cellulose chains,providing more ends for the cellobiohydrolases to act upon; *Exoglucanases*or *cellobiohydrolases*(CBH, 1,4-β -D-glucancellobiodehydrolase, EC3.2.1.91): act on the reducing (CBH I) or nonreducing (CBH II) ends of cellulose chains,liberating cellobiose; andβ -*glucosidases*(β -glucosideglycosyl hydrolase or cellobiase, EC 3.2.1.21): hydrolyzecellobiose or cello-oligosaccharides to glucose and are also involved intransglycosylation reactions of β -glucosidic linkages of glucose conjugates [6]. Cellulase produced by fungi and bacteria, especially by molds, is used extensively in the textile and food industries, bioconversion of lignocellulosic wastes to alcohol, animal feed industry as additive, isolation of plant protoplasts, in plant virus studies, metabolic investigations and genetic modification experiments [7, 8, 9].High cost of cellulase is mainly due to the substrates used in production, and also the slow growth rate of fungi. Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used because the Bacterial cellulase usually lacks one(FPASE) of the three cellulase activities. However, cellulases produced by bacteria are often more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolyzed (feedback inhibition). The greatest potential importance is the ease with which bacteria can be genetically engineered. This is needed especially in order to enhance cellulase production.

Agricultural and agro-industrial wastes are abundant, renewable and inexpensive energy sources. Many countries in the world produce abundant agricultural wastes. Such wastes include a variety of materials such as sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switchgrass, and straws, stems, stalks, leaves, husks, shells and peels from cereals like rice, wheat, corn, sorghum and barley, etc. Accumulation of these waste in large quantities present a serious environmental pollution, but these wastes can be utilized to produce a number of value added products such as food additives, enzymes, organic acids, ethanol and others. The non-use of these materials constitutes a loss of potentially valuable resources.

Therefore, this study was carried out to evaluate the production of cellulase by *Aspergillus niger, Penicillium chrysogenum* and *Trichoderma harzianum* on some agricultural wastes.

2. MATERIALS AND METHODS

Sample collection

Soil, mouldy grains and faeces (from large intestine of cattle) were collected from farm, grain vendors and Abattoir respectively for the isolation of fungi as described previously [10]. The Agricultural wastes (sugarcane bagasse, paper, wheat bran) were collected within Kano municipal council. The samples were oven dried at 65°Cfor24 hours. The substrates were ground and pass through a sieve (about 0.5mm pore size) to obtain the fine powder.

Isolation and Identification of Fungi

Fruiting bodies of the molds from the samples were aseptically taken using swab stick and inoculated on the Sabouraud's dextrose agar (SDA) and potato dextrose agar (PDA) plates to spread spores as described elsewhere[11]. The plates were examined for growth after three days of incubation. Colonies from the parent culture were subcultured after seven days and subcultured until pure cultures were obtained and maintained on slants (PDA). Fungal isolates were identified macroscopically and microscopically using slide culture method as recommended previously [12]. The identified isolates were confirmed using standard reference manuals [13].

Standardization of fungal inoculum

Fungal spore suspension was prepared by scooping spores from slant culture with the aid of a inoculating needle and shaken thoroughly in 20 ml of 0.2% tween 80 solution to obtain spore suspension used for this research. The washed spores were placed on slides and counted under microscope. One microlitre spore suspension contained approximately 1.5×10^6 spores per ml.

Pre-treatment of the agricultural wastes

Mechanical pre-treatment

Reduction of particle size was done to make material handling easier and to increase surface/volume ratio. This was carried out by chopping (sugar cane bagasse), shredding (paper) and grinding all the substrate into fine powders and sieved through a mesh 0.5mm pore size

Alkali pre-treatment of the agricultural wastes

The ground carbon source was pre-treated using the method previously described [14]with modification. The substrates 5g/100ml in separate conical flasks (250ml) were soaked in 5% (w/v) NaOH solution in a ratio of 1:20 to delignify it. This was followed by autoclaving at 121^{0}C for 1 hour. The pre-treated substrates were filtered with muslin cloth and residues was neutralised with 1MHCl and washed with distilled water. The pre-treated substrates were dried in oven at 120°C for 6 hours.

Medium Preparation for Enzyme Production

The fermentation medium or mineral salt medium (MSM) was prepared as described by [13] by dissolving the following salt in distilled water (g/litre): KH_2Po_4, 10.5g; $(NH_4)_2So_4$;10.5g;$MgSO_4.7H_2O$, 0.5g; $CaCl_2$, 0.5g; $FeSO_4.7H_2O$, 0.13g; $MnSO_4.7H_2O$, 0.04g; $ZnSO_4.7H_2O$, 0.04g; $CoCl_2.6H_2O$, 0.0067g; K_2HPO_4, 0.5g; $MnCl_2.4H_2O$, 0.05g; Na_2MO_4, 0.5g;inflasks. Five grams of each of the treated substrateswere added separately to 100ml of MSM and the medium was adjusted to pH 5. The medium was sterilized in an autoclave at 121^0 C for 15 minutes [15].

Production of Cellulase

Medium was inoculated with 1ml of fungi from spore suspension with the aid of micropipette and incubated at 30^{0}C in an orbital shaker at 100 rpm for 216 hrs. Enzyme production was monitored after every 24 hrs throughout the period of incubation. Samples of culture fluids were obtained using sterile pipette after the 24 hours and were centrifuged at 4,000rpm for 10 minutes at room temperature and the supernatant was analyzed for the crude enzyme.

Assay of cellulase Activity

Cellulase activity was determined using spectrophotometer by measuring the increase in glucose concentration from the hydrolysis of β 1-4 glycosidic linkages of cellulose [14].From the fermentation medium 1ml of the broth was taken and centrifuged at 4,000 rpm for 10 minutes, to remove the mycelia and supernatant was used for assaying enzymatic activity. Aliquots (0.5µl) of the supernatant were added into 1ml of glucose oxidase reagent (Randox) and incubated for 15minutes at room temperature. Enzyme activity was determined by taking the absorbance of the suspension using spectrophotometer (JENWAY 6305, UK) at 546nm after

adding 1ml of distilled water. A standard was also prepared by adding 0.5µl distilled water to 1ml glucose reagent. One unit of cellulase activity was defined as the amount of enzyme which released 1 unit of reducing sugar measured as glucose per minute under the assay condition. Therefore:

$$\text{Glucose concentration} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Estimation of glucose released was made by the use of Glucose oxidase kits (Randox, UK). All the measurements were carried out in three independent determinations (triplicate)

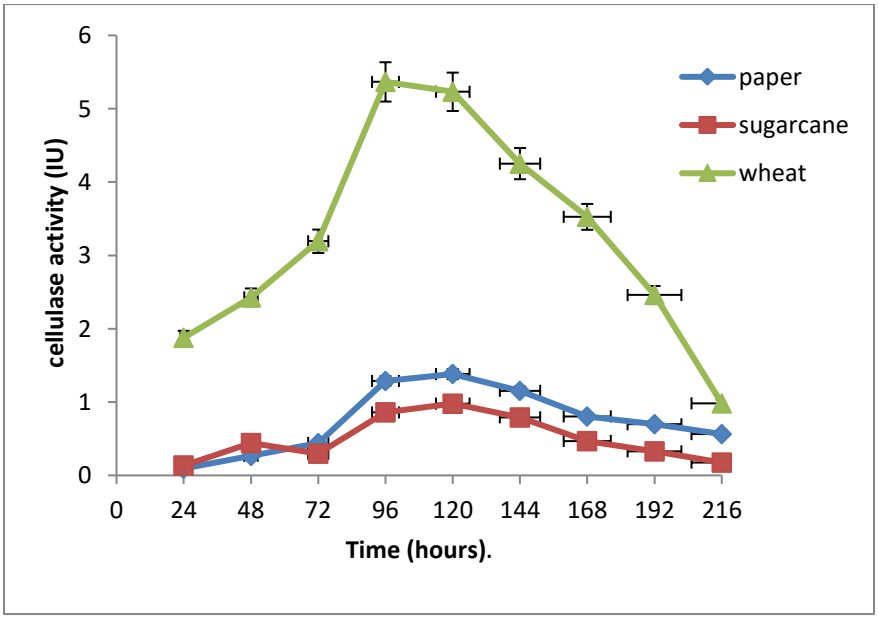
Statistical analysis

Data obtained were analyzed using one way ANOVA and the significant difference was determined using Duncan Multiple Range Test (DMRT) at $P<0.05$ level of confidence.

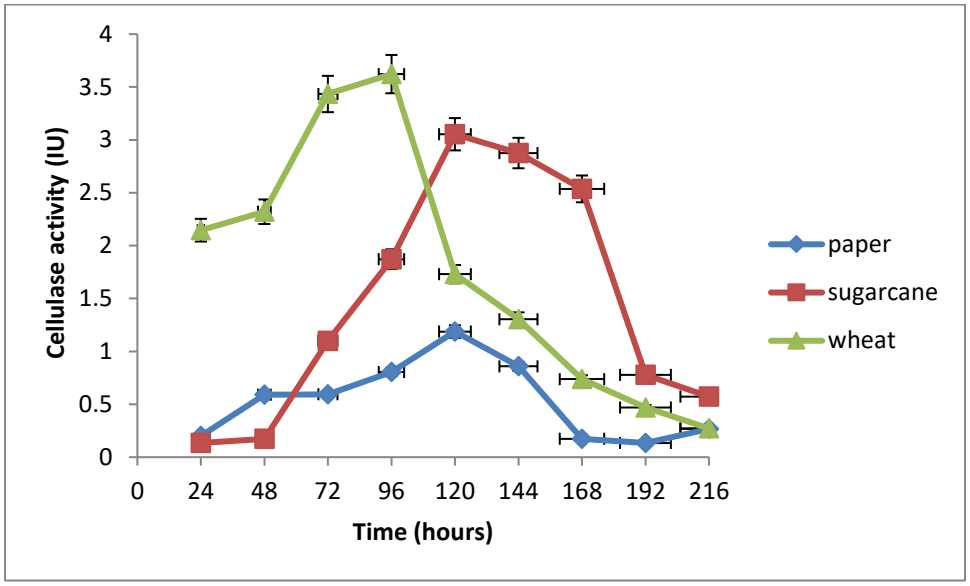
3. RESULTS

A total of three fungal isolates were identified as *Aspergillus niger, Penicillium chrysogenum* and *Trichoderma harzianum* as the commonest and predominant species (Table 1). Cellulase activity was and continued to increase until optimum production was attained as shown in Figure 1a. Higher activity of the enzyme was generally observed between 96 to 120 hours in all the cases. For paper, highest activity (1.383±0.020 IU) was observed at 120 hours and then gradually decreases; for *A. niger*. There was rapid increase in cellulase activity after 72 hours and it reached peak between 96 and 120 hours. With sugarcane bagasse as substrate, the highest activity of cellulase enzymes was 0.979±0.017IU after 120 hours. However wheat bran had the highest activity of cellulase enzyme at 5.366±0.017IU after 96 hours. During the time course assay for cellulase enzyme, culture with wheat bran gave the highest enzyme activity, although with no significant difference ($P<0.05$).

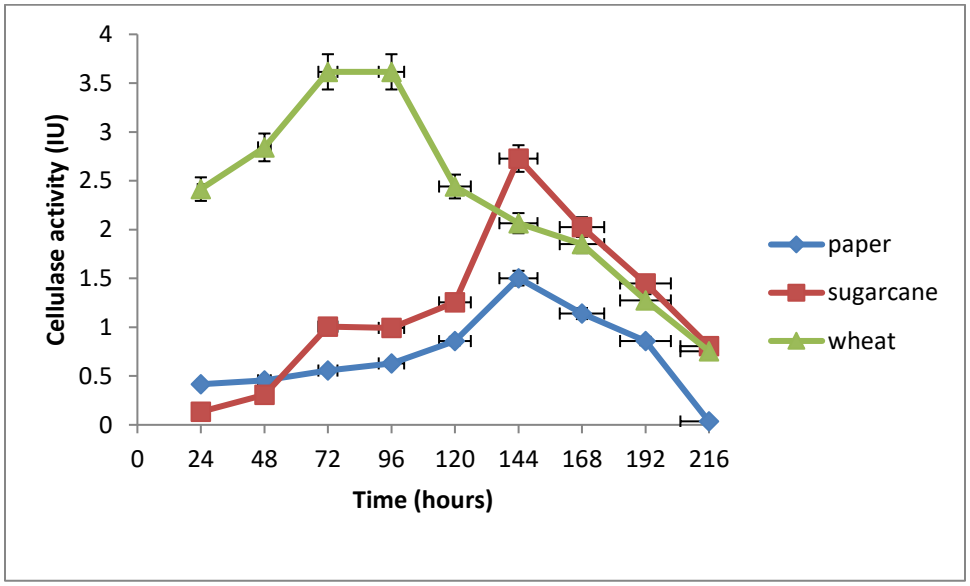
Similarly, using *T. harzianum*(Fig. 1b), Highest enzyme activity produce by paper was at 1.467±0.153IU after 144 hours and gradually decreases up to 216 hours. Culture with sugarcane bagasse had the highest activity of cellulase enzyme at 2.700±0.173IU after 144 hours. Culture with wheat bran had the highest activity of cellulase enzyme at 3.610±0.017IU after 72 to 96 hours. During the time course assay for cellulase enzyme, culture with wheat bran gave the highest enzyme activity also with no statistical significance. Correspondingly, exposure of the substrates to *P. chrysogenum* showed similar outcome with those of the other organisms. Wheat bran was also shown to support more cellulase production, followed by sugarcane bagasse and paper as shown in Figure 1c.



(a)



(b)



(c)

Figure 1 Cellulase activity using (a) *A. niger,* (b) *T. harzianum* and (c) *P. chrysogenum*

Table 1 Characteristics of identified isolates

Macroscopic observation	Microscopic observation	Fungi identified
Compact white basalt felt with a dense layer of dark brown to black colouration was examined on the surface of the plate.	Conidial heads radiate tending to split into loose column with age, vesicles are globose to subglobose. Conidiophores stiped the smooth walled.	*Aspergillus niger*
Colonies grown rapidly in shades of green, sometimes white, mostly dense felt conidiophores colour at first yellow to green or pale to green blue, changing to darker shades, exudates limited or absent.	Conidiophores arising from thesubstrate,mononematous, usaually,ter to quaterverticillate in some strains more stage branched. Conidia at first subglobose to ellipsoidal, remaining so or later becoming globose.	*Penicillium chrysogenum*

4. DISCUSSION

Aspergillus niger, Trichoderma harzianum and *Penicillium chrysogenum* were isolated from spoiled grains for this research. These fungi have been isolated from diverse natural sources, and their enzymes have been characterized for industrially desirable products[18].They are also well known agents of decomposition of organic matter in general and of cellulosic substrate in particular as reported [16]. A wide range of *Aspergillus*, *Trichoderma* and *Penicillium* species have been identified to be active cellulase producers [18] and possess all components of cellulase enzyme system [17] which is in agreement with the present study.

Results showed that cultures with wheat bran had the highest activity at 5.36±0.017IU after 96 hours using *Aspergillus niger* followed by sugarcane bagasse with the highest activity at 1.353±IU and 0.976±0.017IU after 120 hours respectively. The period for maximum cellulase production by *Aspergillus niger* observed in this study is in accordance with numerous findings in the area of research [19]. Fungal enzyme production depends upon cultural/environmental conditions during fermentation and genetic make-up of the organism [16]. The differences in the cellulase activity observed between substrates could be attributed to the differences in the chemical composition and concentration of other macromolecules such as lignin and hemicellulose that exists in natural association with cellulose. Lignin which forms a physical seal around cellulose inhibits cellulase from hydrolyzing the cellulose and hence this may affect the cellulase secreted by the organisms as can be observed with the substrates. The substrates used in this study have low lignin contents in addition to alkaline pretreatment and thus night have increased enzyme activity when compared with other substrates. This observation disagrees with the previous findings [18] that reported a low cellulase activity using *Aspergilllus flavus* with sugarcane bagasse as substrates. Another factor that may lead to the differences in cellulase activity among the substrates is the production of non-specific byproducts, other than glucose by unspecified reactions. These byproducts promote glucose degradation and reduce its yield.

The decrease in cellulase activity shown by the fungi after attaining the highest peak of enzyme secretion could be attributed to so many factors. The end products of cellulase action on cellulose (cellobiose and glucose) inhibit enzyme secretion [22]. Depletion of carbon and nitrogen sources causes starvation and hence the fungi may not grow and cellulase activity is growth related as reported by [22]. The submerged fermentation culture methods used in this study may also contribute to the differences in cellulase activity. A comparison of solid state fermentation and submerge fermentation has shown that submerged method has shearing forces which force rupture of mycelial cells and deactivates enzymes, thus enzyme activity is decreased.

Cellulase production by *A. niger* was observed to be high after 72hours using wheat bran, although the enzyme production declined immediately after 96hrs (4days). However, using paper and sugar cane bagasse, highest production was recorded at 120hrs (5 days) with 1.38±0.020 IU and 0.98±0.015 IU respectively. Enzymes have been reported to have optimum activity at 96 hours substrate exposure. This is in accordance with the previous findings [23]that reported highest enzyme activity after 96 hours using rice bran, with enzyme activity expressed as ratio of yield to time. The decrease in activity in paper and sugarcane bagasse substrates after fermentation period of the highest activity may be attributed to cumulative effects of cellobiose[24]. Cellobiose a dimmer of glucose is known to inhibit both endoglucanase and glucosidase (cellulase). It may also suggest that delignification produces aromatic water soluble products which repress the cellulytic action of the enzymes [14]. Substrate concentration of 5% was shown to support optimum enzyme activity. This can be explained to be as a result of availability of more cellulose at 5% concentration. A decrease in enzyme activity beyond optimum concentration (5%) of substrate may be due to availability of active binding sites or inhibitors. This is supported by the previous findings [24] that reported the inhibitory effect of accumulated cellobiose and cellodextrin of low degree polymerization. The decrease may also be due to depletion of other nutrients (mineral salts) in the medium, other than the energy source or due to the specific binding of the enzymes with the substrates as observed previously [25,26].

5. CONCLUSION

In this study, agricultural wastes were observed to be good substrates that can support production of cellulase enzyme. Fungal species like *A. niger, T. harzianum* and *P. chrysogenum* were able to suitably produce the cellulase enzyme in large amounts, especially between 96 to 144 hours period of exposure. *A. niger* and *P. chrysogenum* were shown to produce more cellulase using wheat bran as substrate and sugarcane bagasse. It is evident from this study that, there is a possibility of utilizing the fungal isolates to produce cellulase enzyme which is a cost effective and environment-friendly, in both small and industrial scale.

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